

# Genetic Diversity of *Spiroplasma citri* Strains from Different Regions, Hosts, and Isolation Dates

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## ABSTRACT

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*Spiroplasma citri*, a phloem-limited pathogen, causes citrus stubborn disease (CSD). Losses due to CSD in California orchards have grown over the past decade. To investigate the possibility of introduction or emergence of a new strain, a study of genetic diversity among *S. citri* strains from various locations was conducted using random amplified polymorphism DNA-polymerase chain reaction (RAPD-PCR) of 35 strains cultured from 1980 to 1993, and of 35 strains cultured from 2005 to 2006. Analysis using 20 primer pairs revealed considerable diversity among

strains. However, no unique genetic signatures were associated with recently collected strains compared with those collected 15 to 28 years ago, and no geographically associated pattern was distinguishable. *S. citri* strains from carrot and daikon radish contain some unique DNA fragments, suggesting some host plant influence. Multiple strains from single trees also showed genetic diversity. Sequencing of five RAPD bands that differed among strains showed that diversity-related gene sequences include virus fragments, and fragments potentially encoding a membrane lipoprotein, a DNA modification enzyme, and a mobilization element. No differences in colony morphology were observed among the strains. The lack of correlation between PCR patterns and isolation date or collection site is inconsistent with the hypothesis that recent infections are due to the introduction or emergence of novel pathogen strains.

Oranges and other citrus fruits are among the most popular fruits in the United States, and California is the major citrus producer for the nation's fresh fruit market (29). California citrus orchards have been affected by citrus stubborn disease (CSD) since 1915 (4). Initially attributed to a viral agent (10), in 1972 the etiologic agent was confirmed as a wall-less bacterium, *Spiroplasma citri* (15,31). Severely affected trees are stunted and have short leaf internodes, leaf mottling, unseasonal blossoms, and lopsided fruits (4).

*S. citri*, a phloem-limited mollicute, is transmitted in a circulative-propagative manner by several species of leafhoppers (21,27). The primary vector of *S. citri* in the United States, *Circulifer tenellus* (Baker), is a polyphagous insect able to transmit the spiroplasma from several weed species commonly found in the foothills of the San Joaquin Valley, CA (3). The general distribution of CSD-infected plants in commercial crops suggests a migration of the infected insects from the weeds to the commercial crops. The ability to feed on different plant species and to migrate long distances make this insect a key element in disease epidemiology (12). Besides *C. tenellus*, *Scaphytopius nitridus* (DeLong) also was reported as vector of *S. citri* (16,27) but its importance in CSD epidemiology remains unclear.

Although CSD has been present in the San Joaquin Valley for many years, its impact in the region apparently increased after a series of freezes in California citrus orchards during the 1990s, as more growers reported CSD symptoms. *S. citri* also was detected for the first time in carrots, first in Washington State (19) and shortly thereafter in California (this paper), possibly reflecting

niche expansion and adaptation of the pathogen to a new plant host.

The very small genome of *S. citri* easily acquires or deletes genetic components, thereby enhancing its fitness (23). For example, continuous graft transmission of *S. citri* from periwinkle to periwinkle resulted in a chromosomal inversion and genomic deletions in *S. citri* BR3-3X that were associated with loss of transmissibility by the natural vector, *C. tenellus* (38,40). High passage in artificial medium also altered *S. citri* transmissibility (38).

Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), using low stringency conditions (22) and random primers having short nucleotide sequences has been used efficiently to discriminate genetic diversity among some plant pathogenic bacteria, including *S. citri* strains (24). Although the reproducibility of RAPD fingerprints can be influenced by the reagents, thermocycler (37) and intensity of amplicons used to score the fingerprint (35), under well-established parameters the results can be very reproducible within a laboratory.

Repetitive element PCR (rep-PCR), which amplifies DNA sequences between repetitive sequences on the bacterial chromosome, also is used to assess genetic diversity in plant pathogenic bacteria (22). The effectiveness of the common rep primers ERIC and BOX, however, apparently is limited in assessing genetic diversity of *S. citri* (25).

The *S. citri* genome has been shown to evolve over relatively short periods of time (23). The possibility that the apparent increase in CSD incidence in California orchards is due to the occurrence of a new *S. citri* strain, led us to compare the genetic diversity among *S. citri* strains recently cultivated from different plant hosts in California (2005 to 2006) with *S. citri* strains cultivated from various locations between 1980 and 1993.

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## MATERIALS AND METHODS

**Bacterial strains and isolation.** *S. citri* strains were obtained from various locations and in different years (Tables 1 and 2). Thirty-five strains, collected over the past 25 years from sweet orange (*Citrus sinensis* [L.] Osb.), grapefruit (*Citrus paradisi* Macf.), horseradish (*Armoracia rusticana* P. G. Gaertn., B. Mey. & Scherb), peach (*Prunus persica* [L.] Batsch), broccoli (*Brassica oleracea* L.) and the beet leafhopper (*C. tenellus*), were available in J. Fletcher's collection. Thirty-three additional strains were collected during this study by cultivation from *S. citri* infected sweet orange, carrot (*Daucus carota* L.), the weed lamb's-quarter (*Chenopodium album* L.), and daikon radish (*Raphanus sativus* L.) plants from several different locations in the San Joaquin Valley, CA. Sampling occurred during 2005 and 2006 (Table 2, Fig. 1). Two California strains (C189 and S600, Table 2) maintained continuously in planta were obtained from the Citrus Clonal Protection Program (CCPP), University of California, Riverside. A single strain from sweet orange was collected in Israel in 2006. Cultivation was performed using standard procedures in LD8 medium (2,20), and strains were triply cloned and stored at  $-80^{\circ}\text{C}$ . One to six strains per orchard were cultured and, except for one experiment designed to assess within-tree diversity, each strain was obtained from a different plant. Strains were named, in general, according to site location (1 through 12) and plant (A through F) (Table 2). Strains of *S. melliferum*, *S. floricola*, *S. phoeniceum*, and *S. kunkelii* also were included (Table 1).

**DNA isolation.** Spiroplasmas were grown in 25 ml of LD8 broth (20) ( $30^{\circ}\text{C}$ ) to a titer of  $10^8$  cells/ml. Cells were harvested, pellets were resuspended in CTAB buffer, and DNA extraction was accomplished via standard procedures (9). The DNA pellets were dissolved in water and quantified in a spectrophotometer (Nanodrop, ND-1000, Wilmington, DE). The DNA solution was diluted to 4 ng/ $\mu\text{l}$  and stored at  $-20^{\circ}\text{C}$ .

**RAPD-PCR and rep-PCR.** Twenty 10-oligonucleotide primers, chosen arbitrarily (OPA-09, OPA-18, OPB-01, OPB-16, OPC-03, OPC-13, OPH-08, OPN-11, OPQ-06, OPY-05, OPY-15, OPZ-15, OPAW-05, OPAX-02, OPBF-05, OPAF-07, OPA-13, OPA-14, OPA-15, and OPB-20, Operon Technologies, Alameda, CA), were used in RAPD amplifications. PCR mixtures (25  $\mu\text{l}$ ) contained 5.7  $\mu\text{l}$  of autoclaved distilled water, 2.5  $\mu\text{l}$  of GoTaq Flexi buffer (10x) (Promega, Madison, WI), 2.5  $\mu\text{l}$  of  $\text{MgCl}_2$  (25 mM), 4  $\mu\text{l}$  of dNTP mix (1 mM each) (Fisher Scientific, Fair Lawn, NJ), 20 ng of template, and 1.5 units of GoTaq DNA polymerase (Promega). PCRs without DNA template were used as negative controls. Initial denaturation was performed at  $94^{\circ}\text{C}$  (6 min), followed by 40 cycles of  $94^{\circ}\text{C}$  (1 min),  $35^{\circ}\text{C}$  (2 min),  $72^{\circ}\text{C}$  (2 min). PTC-200 thermocycler (MJ Research, Inc., Ramsey, MN) was used for all experiments. Rep-PCR conditions were similar, except that the BOX 1AR primer was used (18), and the initial denaturation was  $95^{\circ}\text{C}$  (2 min), followed by 30 cycles of  $94^{\circ}\text{C}$  (3 s),  $92^{\circ}\text{C}$  (30 s),  $50^{\circ}\text{C}$  (1 min), and  $65^{\circ}\text{C}$  (8 min). PCR products were electrophoresed in 1.5% TAE-agarose at 100 V. Bands were compared with a 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA). Gels were stained with ethidium bromide and visualized using an

TABLE 1. Strains of *Spiroplasma citri* and other *Spiroplasma* species analyzed in this study

No.	Strains/species	Geographical origin	Host	Source/(reference)	Isolation date
1	Ex-Cal	California	Sweet orange	B. Kirkpatrick	1984
2	O1	California	Sweet orange	B. Kirkpatrick	1984
3	O-62	California	Peach	B. Kirkpatrick	1984
4	O-202	California	Peach	B. Kirkpatrick	1984
5	O-15	California	Broccoli	B. Kirkpatrick	1984
6	B105	California	Sweet orange	R. Whitcomb	1983
7	B106	California	Sweet orange	R. Whitcomb	1983
8	CB1	California	Leafhopper	R. Whitcomb (5)	1983
9	GO-3	Coachella Valley, California	Grapefruit	G. Oldfield (isolated by J. Fletcher)	1993
10	GO-5	Coachella Valley, California	Grapefruit	G. Oldfield (isolated by J. Fletcher)	1993
11	BR1	Illinois	Horseradish	J. Fletcher (13)	1980
12	BR6	Illinois	Horseradish	J. Fletcher (13)	1980
13	BR11	Illinois	Horseradish	J. Fletcher	1984
14	BR 12	Illinois	Horseradish	J. Fletcher	1984
15	BR 14	Illinois	Horseradish	J. Fletcher	1984
16	BR 15	Illinois	Horseradish	J. Fletcher	1984
17	BR 17	Illinois	Horseradish	J. Fletcher	1984
18	BR 18	Illinois	Horseradish	J. Fletcher	1984
19	BR3	Illinois	Horseradish	J. Fletcher (13)	1980
20	BR3G	Derived from BR3	Horseradish	J. Fletcher (38)	NA <sup>a</sup>
21	BR3T	Derived from BR3	Horseradish	J. Fletcher (38)	NA
22	BR3-42	Derived from BR3	Horseradish	J. Fletcher (38)	NA
23	BR3-80	Derived from BR3	Horseradish	J. Fletcher (38)	NA
24	BR3P	Derived from BR3	Horseradish	J. Fletcher (38)	NA
25	MDHR2	Charles County, Maryland	Horseradish	R. Davis (7)	1981
26	MDHR4	Charles County, Maryland	Horseradish	R. Davis (7)	1981
27	MDHR5	Charles County, Maryland	Horseradish	R. Davis (7)	1981
28	M200H	Derived from R8A2	Sweet orange	R. Davis (34)	NA
29	M200HX	Derived from R8A2	Sweet orange	R. Davis (34)	NA
30	MR3	Derived from R8A2	Sweet orange	R. Davis (34)	NA
31	R8A2	Morocco	Sweet orange	R. Davis (31)	1985
32	Beni-Mellal	Morocco	Sweet orange	S. Purcell	1984
33	Maroc	Morocco	Sweet orange	R. Whitcomb	1983
34	Iran	Iran	Sweet orange	R. Whitcomb	1983
35	ASP-1	Israel	Sweet orange	R. Davis (36)	1981
36	<i>Spiroplasma melliferum</i> /TS-2	Maryland	Honey bee	R. Davis (5)	1991
37	<i>Spiroplasma phoeniceum</i>	Middle East	Periwinkle	R. Davis	1982
38	<i>Spiroplasma floricola</i> /23-6	Maryland	Tulip tree	R. Davis (6)	1978
39	<i>Spiroplasma kunkelii</i> /CR2	Alajuela, Costa Rica	Corn	J. Fletcher	1987

<sup>a</sup> NA, does not apply.

AlphaImager and AlphaEase FC software (Alpha Innotech Corporation, San Leandro, CA).

Patterns of RAPD and rep-PCR DNA fingerprints were assessed visually. The presence or absence of bands in each strain was transformed into binary data (presence = 1, absence = 0) using an Excel (Microsoft) processor and the data saved as a text file. Data reliability was assessed using SEQBOOT (PHYLIP) (11), followed by the MIX parsimony program (PHYLIP) (11). Consensus trees (Consense, PHYLIP) were generated using *S. floridicola* as outgroup. The tree was visualized in the TREEVIEW program (28). Complementary binary data were analyzed using the SAS/PRINCOMP procedure, SAS software 9.1 (33).

**Within-tree *S. citri* genetic variability.** To assess within-tree spiroplasma variability, three infected sweet orange trees from each of two orchards (orchards 4 and 5, Fig. 1) were selected randomly. On 17 May 2006, one fruit was harvested from each of four different locations within each tree (total of four fruits per tree), and spiroplasmas were cultured in LD8 broth from columella tissue (2,20). Initial culture filtrates were diluted and subcultured on agar-solidified LD8 medium; seven to eight different individual colonies per tree were increased in liquid LD8 broth. DNA extraction and RAPD-PCR were performed as described above using primers OPA-09, OPY-05, OPC-13, and OPB-20, which had previously proved useful to differentiate strains.

**Sequencing of differential bands.** Five differential amplicons were extracted from agarose gels and purified with a GeneClean Turbo Kit (Qbiogene, Inc., Carlsbad, CA). DNA was cloned in *Escherichia coli* (strain Mach 1-T1<sup>R</sup>) using the Topo TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmids from successfully

transformed clones were extracted by a small-scale preparation alkaline lysis and cleaned with chloroform:phenol separation, and dissolved in water (32). The target inserts were confirmed by restriction digests with *EcoRI* according to product specifications (Promega). Sequencing was performed using standard methods in an automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were compared by nucleotide blast and submitted to NCBI data base (1).

***S. citri* colony morphology in LD8 medium.** To assess whether genetic diversity was related to *S. citri* colony morphology, two historical (strains 19 and 35, Table 1) and nine recently collected *S. citri* strains (strains 40, 41, 47, 54, 56, 63, 68, 73, and 74, Table 2) were selected for further study.

Twenty-four hour old cultures of *S. citri* strains were diluted in PBS buffer and plated on LD8 agar (0.6 or 1.0% noble agar). Colony morphology was evaluated after 10 or 30 days of incubation at 30°C in the dark. Dienes' stained (8) plates were observed using stereo and light microscopy. Images were acquired in bright field using an Olympus BX51 microscope (Olympus Optical Co., Tokyo, Japan). From strains that yielded more than one type of colony, single cells were subcultured in LD8 medium to assess the stability of the colony morphology. This experiment was repeated two times.

## RESULTS

**RAPD-PCR and rep-BOX.** All 20 RAPD primers yielded differential amplification patterns among the five spiroplasma species evaluated, and 17 did so among *S. citri* strains. Patterns

TABLE 2. *Spiroplasma citri* strains cultivated in 2005 and 2006 and analyzed in this study

No.	Site <sup>a</sup>	Strains		Origin	Host	Source/reference	Isolation
		This paper designation	Original designation				
40	11	C5	C5	Kern County, California	Carrot	This study	2005
41	11	C17	C17	Kern County, California	Carrot	This study	2005
42	2	2A	Ca 1	Tulare County, California	Sweet orange	This study	2005
43	3	3A	Ca 7	Tulare County, California	Sweet orange	This study	2005
44	3	3B	Ca 12	Tulare County, California	Sweet orange	This study	2005
45	3	3C	Ca 16	Tulare County, California	Sweet orange	This study	2005
46	3	3D	Ca 20	Tulare County, California	Sweet orange	This study	2005
47	3	3E	Ca 23	Tulare County, California	Sweet orange	This study	2005
48	3	3F	Ca 28	Tulare County, California	Sweet orange	This study	2005
49	6	6A	Ca 35	Kern County, California	Sweet orange	This study	2005
50	6	6B	Ca 40	Kern County, California	Sweet orange	This study	2005
51	6	6C	Ca 42	Kern County, California	Sweet orange	This study	2005
52	6	6D	Ca 46	Kern County, California	Sweet orange	This study	2005
53	6	6E	Ca 51	Kern County, California	Sweet orange	This study	2005
54	6	6F	Ca 53	Kern County, California	Sweet orange	This study	2005
55	4	4A	Ca 62	Kern County, California	Sweet orange	This study	2005
56	NA <sup>b</sup>	Ir	Israel	Bet-Dagan, Israel	Sweet orange	This study	2006
57	1	Dk	Ca 264	Fresno County, California	Daikon radish	This study	2005
58	4	4B	Ca 204	Kern County, California	Sweet orange	This study	2006
59	4	4C	Ca 198	Kern County, California	Sweet orange	This study	2006
60	4	4D	Ca 207	Kern County, California	Sweet orange	This study	2006
61	5	5A	Ca 160	Kern County, California	Sweet orange	This study	2005
62	5	5B	Ca 242	Kern County, California	Sweet orange	This study	2006
63	5	5C	Ca 256	Kern County, California	Sweet orange	This study	2006
64	NA	C189	C189	CCPP <sup>c</sup>	Sweet orange	CCPP (15)	2006
65	NA	600B	600B	CCPP	Sweet orange	CCPP	2006
66	9	W	Ca 263	Fresno County, California	Lamb's-quarter	This study	2006
67	7	7A	Ca 144	Kern County, California	Sweet orange	This study	2005
68	8	8A	Ca 170	Fresno County, California	Sweet orange	This study	2006
69	12	C1	C1	Kern County, California	Carrot	This study	2006
70	12	C3	C3	Kern County, California	Carrot	This study	2006
71	12	C6	C6	Kern County, California	Carrot	This study	2006
72	12	C7	C7	Kern County, California	Carrot	This study	2006
73	12	C8	C8	Kern County, California	Carrot	This study	2006
74	10	SL	SL	Kern County, California	Sweet orange	This study	2006

<sup>a</sup> See map (Fig. 1).

<sup>b</sup> NA, does not apply.

<sup>c</sup> CCPP, Citrus Clonal Protection Program, University of California, Riverside.

that revealed the greatest diversity among strains are presented (Figs. 2 and 3). The 159 differential RAPD amplicons ranged from 3.0 to 0.25 kbp in size. No consistent differences were observed between new strains of *S. citri* collected in this study and strains cultured from up to 27 years ago ("historic" strains) (Fig. 2). Most of the amplicon patterns observed from new strains of *S. citri* varied from site to site of geographical origin; however, a few correlated consistently with the site of collection (Fig. 2B, arrow). Some fragments from carrot (Fig. 3A, arrow) or daikon radish (Fig. 3B, star) strains were not amplified from citrus strains. Banding profiles produced by BOX-PCR were indistinguishable among the strains (data not shown).

Transformed binary data from the RAPDs was used to generate a phylogenetic tree (Fig. 4) in which *S. floricola*, *S. phoeniceum*, *S. kunkelii*, and *S. melliferum* formed four separate branches. *S. citri* strains fell into two major clusters, both containing mixtures of historic and new strains. One cluster included new strains from sweet orange orchards 2, 4, 5, and 8, a new strain from Israel, and carrot strains from two different California sites. The other cluster included new strains from sweet orange orchards 3, 6, 7, 10, and from the lamb's-quarter, collection site 9 (Fig. 4).

Spiroplasma strains from carrot were all highly similar to one another and most clustered together (Fig. 4). In contrast, sweet orange strains newly cultured from 8 different groves were widely distributed in the two main brackets of the dendrogram (Fig. 4). Although orchards 3, 4, 5, and SL were adjacent to one another along the Sierra Mountain foothills in Kern County, CA, high variability in RAPD patterns resulted in the placement of strains from these orchards into two separate clusters (Fig. 4). Moreover, significant genetic diversity among spiroplasmas from orchards 4 and 5 was observed; these strains clustered more loosely than did strains from orchards 3 and 6, which were highly similar to one another (Fig. 4).

Both historic and new strains of *S. citri* were distributed widely among branches of the phylogenetic tree, presenting little evidence of correlation between clustering and date of isolation (Fig. 4). Exceptions were strains R8A2, Maroc, MR3, M200HX, and M200H, all of which are from the same geographical region; in fact, the latter three are laboratory derivatives of the first (34). Interestingly, strain BR3, the progenitor of BR3-42, BR3-80, BR3-P, BR3-T, and BR3-G, clustered closely only with BR3-42, which was derived from BR3 by 42 successive subcultures (14). It was somewhat removed from BR3-80 and BR3-P, which were derived from it by 80 or 130 successive subcultures, respectively (38), and from BR3-T and BR3-G, which were derived from it by successive transmission by beet leafhoppers or periwinkle grafting, respectively (38). In addition, *S. citri* strains BR15, BR17, and BR18, from adjacent horseradish field sites in Illinois, also showed significant genetic diversity (Fig. 4).

Principal component analysis (PCA) of *S. citri* historic and new strains showed that the first two principal components explained 30.3% of the variation encountered in the analysis. Primers OPA-15, OPA-18, and OPN11 appeared to have a great influence on the first two components. In agreement with the phylogenetic tree, there was no clustering between historic and new strains (Fig. 5A). PCA of *S. citri* strains cultivated from orchards 3 to 6 showed that in some orchards the genetic variability within a grove was very limited and in others the genetic variability within groves was greater (Fig. 5B).

**Within-tree genetic diversity.** *S. citri* genetic diversity within single sweet orange trees in orchards 4 and 5 was evidenced by differential banding patterns obtained using primers OPA-09 and OPC-13. In orchard 4, only one of the three sweet orange trees evaluated yielded multiple genotypes (data not shown). In orchard 5, two of the three plants evaluated were infected by more than one type of spiroplasma in the same plant. One of the trees yielded two, and the other tree yielded three, different RAPD patterns (Fig. 6).

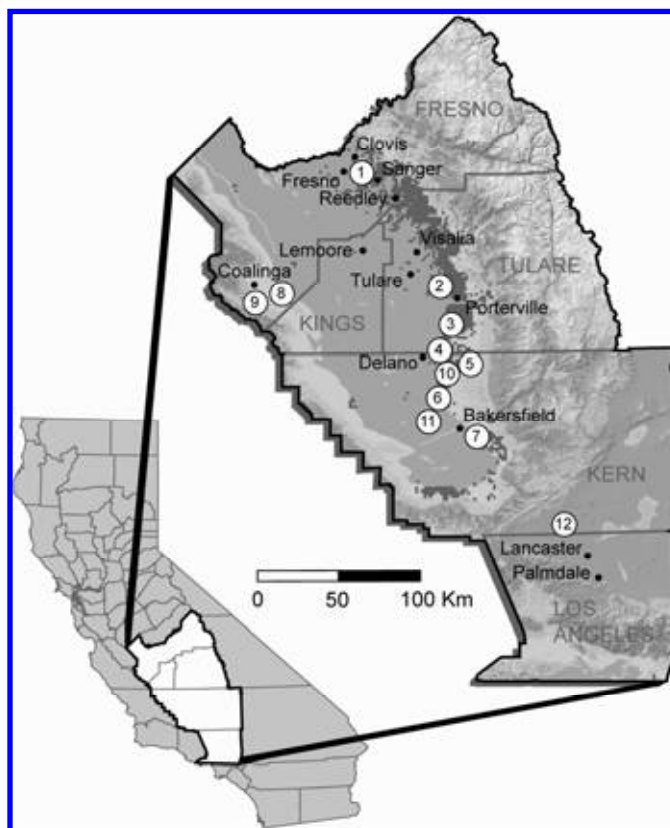
**Sequencing of differential amplicons.** Sequencing of five RAPD amplicons that were produced differentially among strains (diversity-related gene sequences) identified two plectroviral fragments and fragments potentially encoding parts of a membrane lipoprotein, a DNA modification enzyme, and a mobilization element (Table 3).

***S. citri* colony morphology.** Of the 11 *S. citri* strains plated onto solid medium, only strain 35 (ASP-1) presented a stable colony morphology in both experiments performed, independent of the agar concentration used; its colonies were of the "fried egg" type (colony type B, Fig. 7). All other strains produced some colonies with fried egg morphology and others having a dense center surrounded by satellite colonies (colony type A, Fig. 7). When colonies of either type were picked, subcultured in LD8 broth medium and re-plated, they again yielded colonies of both types (Fig. 7).

## DISCUSSION

Genetic diversity in bacteria can be assessed by examining specific restriction sites, repetitive elements, genome sequences, or the amplicons produced by random primers (RAPD) (22). RAPD-PCR, when optimized for a particular application, provides effective discrimination among species and strains (30). RAPD primers used in the present study were suitable for differentiating *S. citri* from other members of spiroplasma serogroup I (*S. melliferum*, *S. kunkelii*, and *S. phoeniceum*) and serogroup III (*S. floricola*) and also for discriminating genetic differences among *S. citri* strains.

Significant genetic variability of *S. citri* apparently has existed in California, Maryland, and Illinois for a long period of time. Strains obtained in 1993 (GO3 and GO5) from two grapefruit trees in the same Coachella Valley, CA, orchard and located only

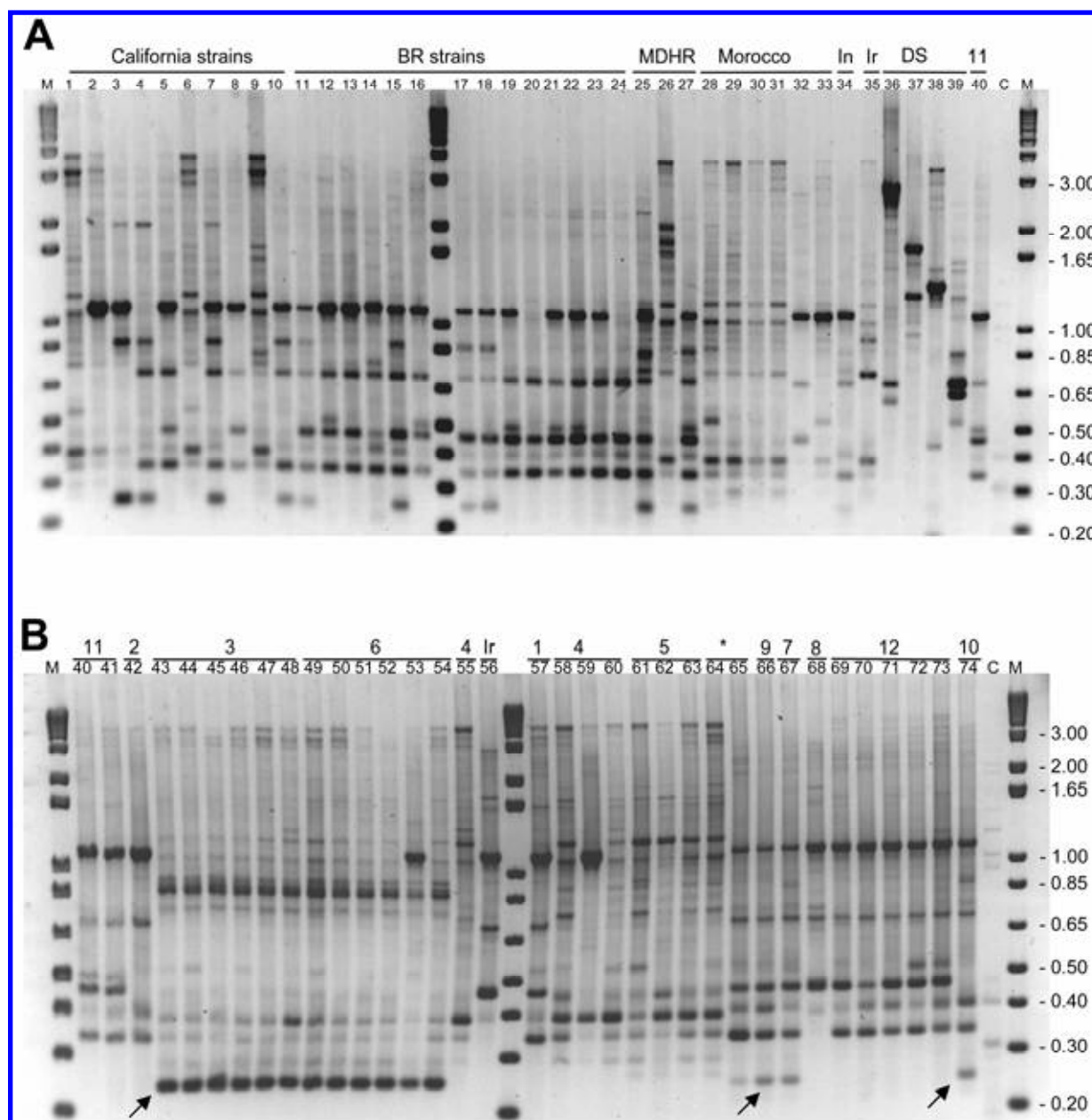


**Fig. 1.** Location of citrus orchards and other sites in the San Joaquin and Antelope Valleys, CA where *Spiroplasma citri* was collected for this study. Each gray square represents 1.61 km<sup>2</sup> in which a commercial orchard of 25 or more citrus trees was planted.

two trees apart, yielded so many differential amplicons that these strains were separated into the two main branches of the phylogenetic tree. The same phenomenon was observed with some *S. citri* strains cultivated from 1981 to 1984 from horseradish plants with symptoms of brittle root disease from adjacent fields in Illinois or Maryland (7,13). Yokomi et al. (41) have shown the presence of two genetically different populations of *S. citri* in field trees from central California and from historical strains collected from southern California in the 1960s.

It is challenging to evaluate the significance of the differences among strains and clusters in the RAPD-PCR generated phylogenetic tree of Figure 4. One measure of significance can be generated by examining two cases in which several different *S. citri* lines, derived from a common parent strain under laboratory conditions, generated different RAPD patterns in the present work. Such comparisons are useful because, since we know the deriva-

tion histories and some of the genomic information about these strains, we can apply that information to inform our interpretation of the RAPD results in this study. In one example, strain BR3, which was cultivated originally from *S. citri*-infected Illinois horseradish, generated derivative genotypes after successive subculturing in vitro or after extended cycles of either leafhopper transmission or plant graft transmission (14). Previous work revealed differences in protein profiles of the nontransmissible BR3-derivative lines BR3-P and BR3-G, obtained by in vitro and graft transmission, in comparison with BR3, the parent line, and with BR3-T, a still-transmissible leafhopper-transmitted derivative (14). In addition, BR3-G had sustained a chromosomal inversion and two deletions compared with its progenitor (40), mutations that likely were involved in its loss of transmissibility. In the lineage proposed by our RAPD analysis (Fig. 4), BR3 and BR3-T are each more closely aligned with other *S. citri* strains than to



**Fig. 2.** Random amplified polymorphism DNA (RAPD) fingerprints generated using primer OPA-15 and a template of historic (A) or new strains (B) from California and Israel. Numbers below bars indicate strain; lanes C: the control reaction without template DNA, lanes M: DNA ladder 1 kb plus; size fragments are listed on the right. Above the bar: **A**, Historic strains: 1 to 10, from California; 11 to 24, from Illinois horseradish; 25 to 27, from Maryland horseradish; 28 to 33, from Morocco; 34 and 35, from Iran (In) and Israel (Ir); 36 to 39, different species of spiroplasma (DS), and 40, new strain from site 11. **B**, New strains: site 1, daikon radish; sites 2 to 8 and 10, citrus orchards; site 9, lamb's-quarter; and sites 11 and 12, carrot. Asterisk indicates strains cultivated from grafted plants from the Citrus Clonal Protection Program; and Ir, new strain from Israel. Arrows indicate *Spiroplasma citri* amplicon specific to some citrus sites and to lamb's-quarter.



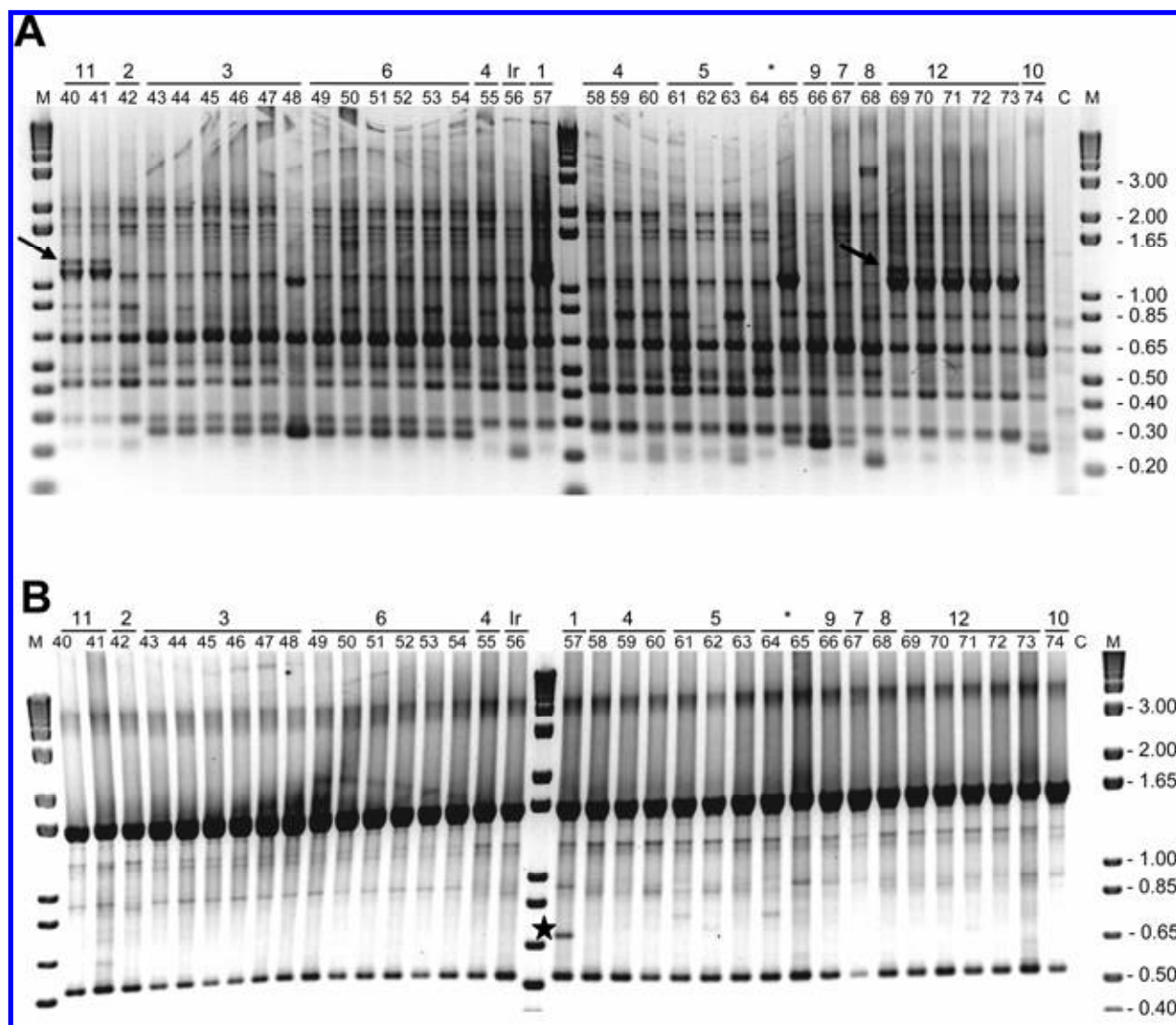
each other. In a second example, RAPD pattern differences were observed among strains M200H, M200HX, and MR3, all of which are subculture derivatives of strain R8A2. MR3 is able to resist infection by a spiroplasma virus isolated from *S. melliferum*, unlike its parent, which remains susceptible (34). Consideration of the phylogenetic tree clusters in tandem with previous knowledge of the same strains provides a sense of the scale of the differences identified in this study.

Despite the great variability in RAPD profiles encountered among the 70 *S. citri* strains evaluated in this study, no particular pattern differentiates *S. citri* strains collected between 2005 and 2006 from strains collected between 1980 and 1993. Comparison among strains recently collected from California orchards showed that genetic patterns of *S. citri* within an orchard could be similar or very different. Since the majority of the orchards sampled were located near the foothills in the east side of the Central Valley, the different degrees of genetic diversity may reflect greater or lesser exposure of the trees to insect vectors, and, consequently, more or fewer inoculation events. Genetic homogeneity in some groves could also be consequence of dispersion by nursery propagation. One RAPD primer (OPA-13) differentiated between carrot and citrus strains of *S. citri*. This result is different from an earlier

report that showed no significant genetic differences among citrus and carrot strains. However, since the first study used the 16S rDNA region that is used primarily as a bacterial taxonomic tool these RAPD results are not comparable (19).

The *S. citri* genome is one of the largest among Mollicutes, and is characterized by high adenine-thymine content. In addition to its circular chromosome, *S. citri* also harbors plasmids and virus genomes, which likely serve as sources of genetic information (23,39). Genetic variations of spiroplasmas are attributed to DNA acquisition and loss, DNA replication and repair, homologous recombination and transposition (23). Amplicons sequenced in the present work showed that, in addition to the fact that bacteriophages occupy a considerable proportion of the *S. citri* genome, genetic differences among strains may result from several different mechanisms within the genome. Such inserts included a hypothetical gene for a DNA methyltransferase, an enzyme associated with DNA protection and repair, a transmembrane lipoprotein which the C-terminal is also virtually identical to SpV1-R8A2 B phage ORF product and a mobilization-like protein.

*S. citri* cells usually are motile and helical when cultured on LD8 medium, and move linearly in medium solidified with a low

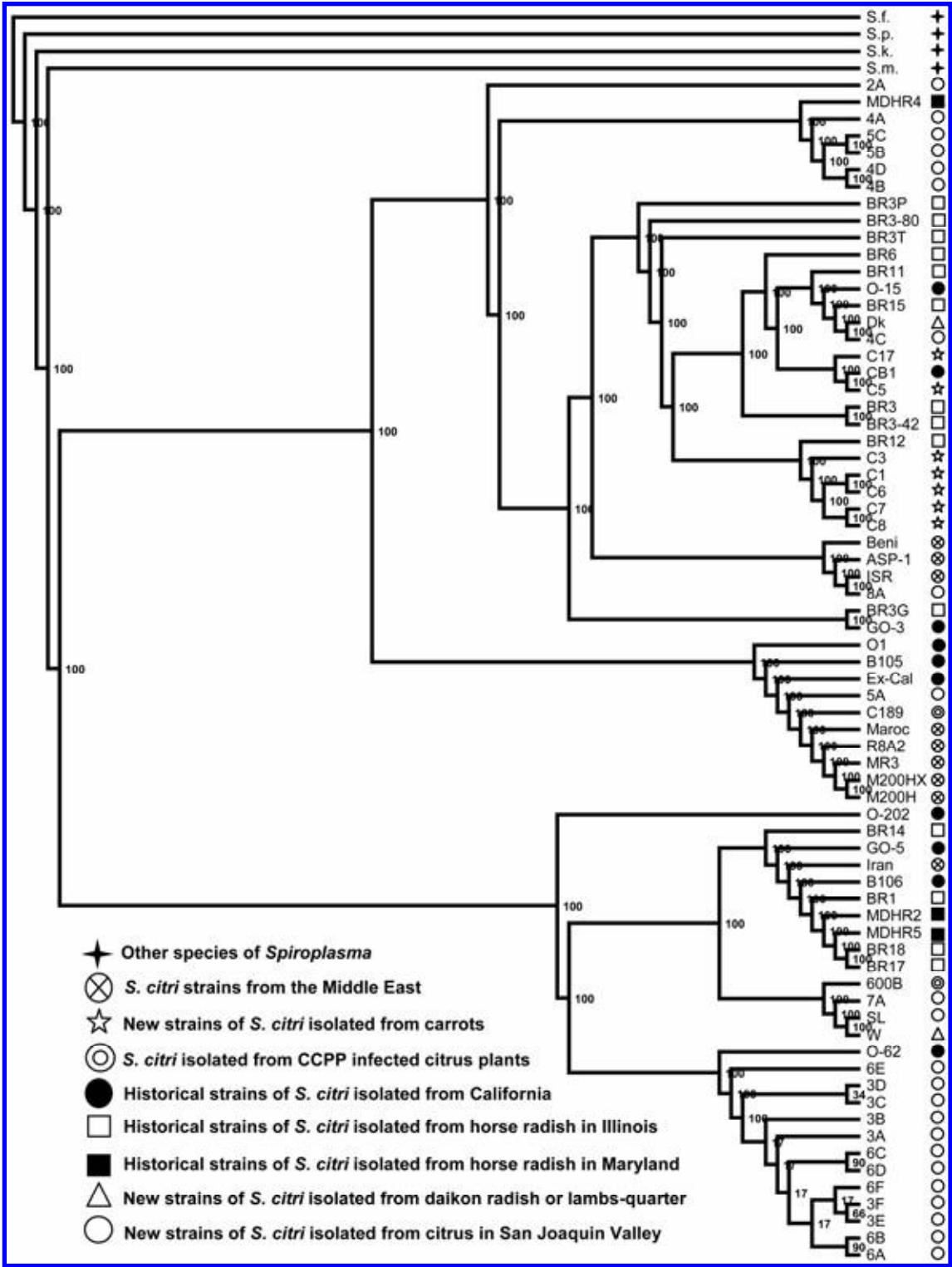


**Fig. 3.** Random amplified polymorphism DNA fingerprints after amplification with primers **A**, OPA-13 and **B**, OPZ-15; template DNA from new strains from California and Israel. Numbers below the bar show strain identification; lanes C: the control reaction without template DNA; lanes M: DNA ladder 1 kb plus, size fragments on right. Above the bar: site 1, daikon radish; sites 2 to 8 and 10, citrus orchards; site 9, lamb's-quarter; sites 11 and 12, carrot; Asterisk indicates strains cultivated from grafted plants obtained from Citrus Clonal Protection Program; and Ir, new strain from Israel. Arrows indicate a specific amplicon in carrots (40, 41 and 69 to 73) (**A**) and the star indicates a specific amplicon in daikon radish (57) (**B**).

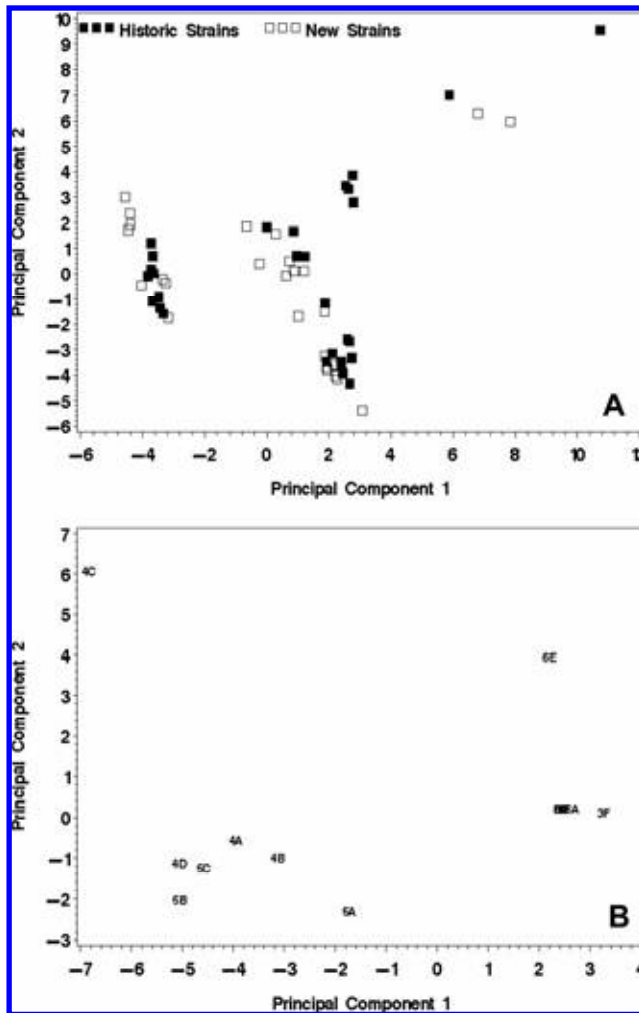
agar concentration, forming colonies with indistinct edges due to the formation of satellite colonies. Strains having low motility in agar medium yield colonies with well-defined edges, sometimes called “fried-egg” colonies. These colony types have been found in natural populations from CSD citrus trees in the Middle East (36), and also in motility-impaired mutants generated by Tn4001 transposition (17). In our study, except for *S. citri* ASP-1, previously reported as a nonhelical, nonmotile, fried-egg colony-producing strain, none of the spiroplasma strains maintained a

single stable colony morphology during successive subculturing. Colony morphology apparently is unreliable as a means of differentiating strains. Whether such variation is related to the phenomenon of phase variation, in which a variety of molecular mechanisms in mollicutes give rise to variable phenotypes (26), is not known.

*S. citri* genetic diversity within single trees in citrus orchards 4 and 5 showed that, under conditions of commercial citrus orchards, populations of *S. citri* may be heterogeneous. The explanation for



**Fig. 4.** A phylogenetic tree constructed by mix parsimony (PHYLIP) based on the 159 characters of the 70 different strains of *Spiroplasma citri* and of *S. melliferum* (S.m), *S. floricola* (S.f.), *S. phoeniceum* (S.p.), and *S. kunkelii* (S.k). Numbers at the nodes indicate the levels of bootstrap support (percentage) based on 1,000 repetitions for the branches immediately to the left.



**Fig. 5.** Arrangement of *Spiroplasma citri* strains based on principal component analysis using as input the differential characters obtained in random amplified polymorphism DNA reactions. **A**, Analysis including historic *S. citri* strains (black squares) and new strains (open squares). **B**, *S. citri* isolates from orchards (sites) 3 to 6. Majority of strains from orchards 3 and 6 formed a tight cluster at principal coordinate 1 (3) and principal coordinate 2 (0).

such diversity could be multiple natural infections of single trees by heterogeneous strains, a series of in planta genetic alterations, or grafting of young rootstocks with mixtures of field-infected and nursery-contaminated bud-wood.

The lack of major differences among historic and new strains leads us to conclude that genetic changes in *S. citri* genome were not a significant factor in any reemergence of CSD in California orchards. The epidemiology of CSD is complex. The pathogen infects several plant host species including commercial crops and weeds, and has several different leafhopper vectors, all of which could influence the impact of CSD in California.

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**TABLE 3.** Description and putative functions of sequences of strain-differential amplicons of *Spiroplasma citri* obtained by random amplified polymorphism DNA-polymerase chain reaction

Strain sequenced	Amplicon size (bp)	Accession number	Function/product
35 <sup>a</sup>	520	EU005544	Plectrovirus Spv1-c74
68 <sup>b</sup>	176	EU005545	Plectrovirus Spv1-R8A2b
43 <sup>c</sup>	227	EU005546	Hypothetical lipoprotein transmembrane
40 <sup>d</sup>	1186	EU005547	MOB-like protein
63 <sup>e</sup>	688	EU005548	Hypothetical DNA methyltransferase protein

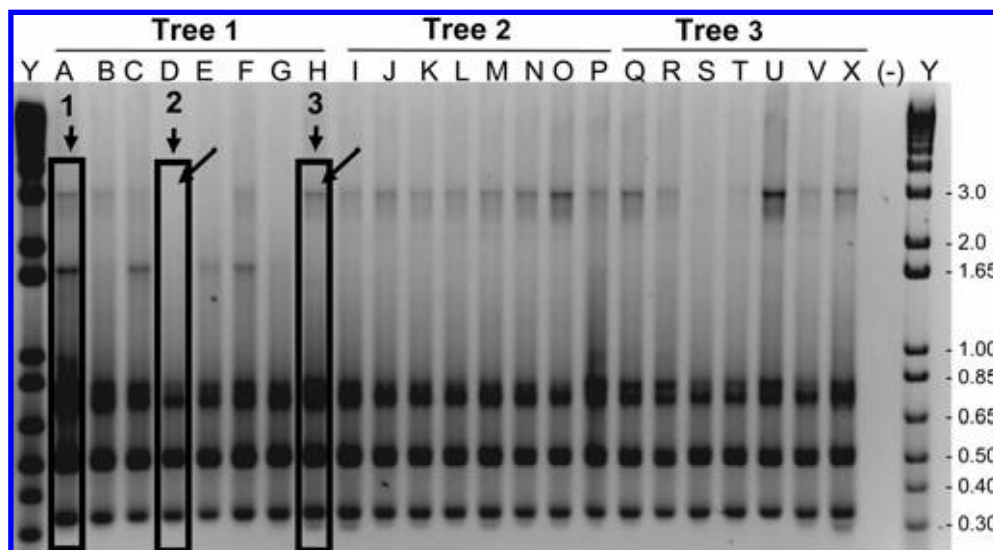
<sup>a</sup> Amplicon unique to historic strains 19, 22, 24, 34, and 35; the new Israeli strain 56; and a new strain from site 8 in California, 68, using primer OPA-09.

<sup>b</sup> Amplicon present in new strains 40 to 42, 57 to 63, 66, and 68; and historic strain 35, using primer OPA-13.

<sup>c</sup> Amplicon present only in trees from sites 3, 6, 7, and 9 (new strains 43 to 54 and 65 to 67) and in historic strains 3, 7, 10, 11, 15, 17, 18, 25, and 27, using primer OPA-13.

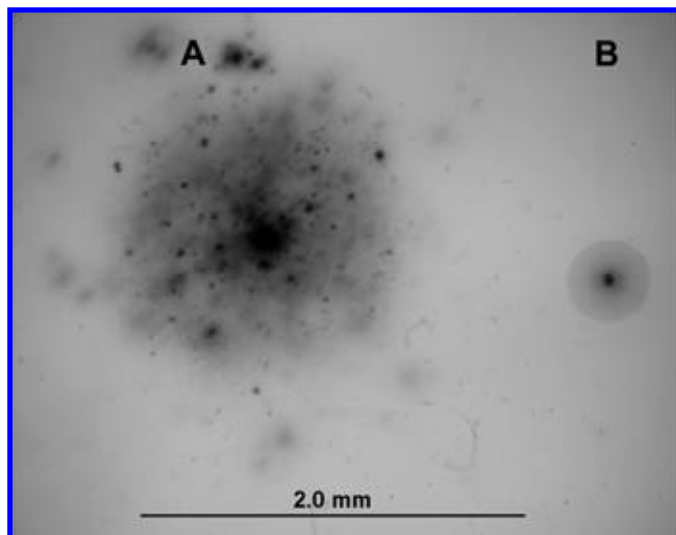
<sup>d</sup> Amplicon present only in carrot strains 40, 41, and 69 to 73, daikon radish strain 57, and historic strains 5, 8, 11 to 14, 16 to 19, 21 to 24, and 26, using primer OPA-13.

<sup>e</sup> Amplicon present in some, but not all, trees of site 6, using primer OPC-13.



**Fig. 6.** Random amplified polymorphism DNA fingerprints after amplification with primer OPA-09. DNA extracted from 7 to 8 *Spiroplasma citri* strains, selected by single-cell cloning from multiple fruits from three different sweet orange trees in orchard 5, served as template. Above the bar: tree identification; below the bar: strain identification; tree 1, strains A to H; tree 2, strains I to P; tree 3, strains Q to X and the control reaction without template DNA (-). Lane Y: DNA ladder 1 kb plus, size fragments listed on right. Arrows indicate differential patterns: 1, presence of 3.0 and 1.65 kbp amplicons; 2, absence of 3.0 and 1.65 kbp amplicons; and 3, presence of 3.0 kbp and absence of 1.65 kbp amplicons.





**Fig. 7.** Mixed colony types of *Spiroplasma citri* obtained from strain 41, cultivated from infected carrot plants on agar-solidified (0.6%) LD8 medium. **A**, colony with satellites and **B**, fried egg colony.

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